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AAV8-mediated gene transfer of microRNA-132 improves beta-cell function in mice fed a high fat diet

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2. Abstract

MicroRNAs have emerged as essential regulators of beta-cell function and beta-cell proliferation. One of these microRNAs, miR-132, is highly induced in several obesity models and increased expression of miR-132 *in vitro* modulates glucose-stimulated insulin secretion. The aim of this study was to investigate the therapeutic benefits of miR-132 overexpression on beta-cell function *in vivo*. To overexpress miR-132 specifically in beta-cells, we employed adeno-associated virus (AAV8) mediated gene transfer using the rat insulin promoter in a double-stranded, self-complementary AAV vector to overexpress miR-132. Treatment of mice with dsAAV8-RIP-mir132 increased miR-132 expression in beta-cells without impacting expression of miR-212 or miR-375. Surprisingly, overexpression of miR-132 did not impact glucose homeostasis in chow fed animals. Overexpression of miR-132 did improve insulin secretion and hence glucose homeostasis in high-fat diet fed mice. Furthermore, miR-132 overexpression increased beta-cell proliferation in mice fed a high-fat diet. In conclusion, our data show that AAV8-mediated gene transfer of miR-132 to beta-cells improves beta-cell function in mice in response to a high fat diet. This suggests that increased miR-132 expression is beneficial for beta-cell function during hyperglycemia and obesity.

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3. Introduction

Decreased beta-cell function plays a pivotal role in the development of type 2 diabetes mellitus. Impaired beta-cell function is an early step in the course of type 2 diabetes mellitus, and the onset of beta-cell dysfunction seemingly occurs long before the development of hyperglycemia (Perley & Kipnis 1967; Kahn *et al.* 2001). MicroRNAs (miRNAs) are a recently discovered class of evolutionarily conserved short noncoding RNAs that regulate gene expression at a posttranscriptional level. MiRNAs bind with imperfect complementary to 3'-UTRs of target mRNAs, causing translational repression of the target gene or degradation of the target mRNA (Bartel 2004). MiRNAs are involved in a wide range of processes that includes development, apoptosis, proliferation, differentiation and regulation of metabolism. In beta-cells, miRNAs have emerged as essential regulators of beta-function, beta-cell proliferation and beta-cell survival (Poy *et al.* 2009; Latreille *et al.* 2014; Tattikota *et al.* 2014; Belgardt *et al.* 2015).

Obesity, a major risk factor for type 2 diabetes mellitus, is known to change miRNA expression in islets (Zhao *et al.* 2009; Nesca *et al.* 2013). One of these miRNAs, miR-132, is of particular interest as expression of this miRNA is highly induced in islets of several obesity models (Zhao *et al.* 2009; Esguerra *et al.* 2011; Nesca *et al.* 2013). This obesity-related increased expression is severely reduced in diabetic-susceptible BTBR ob/ob mice (Zhao *et al.* 2009). Overexpression of miR-132 in rodent beta cells *in vitro* results in enhanced glucose-induced insulin secretion (Nesca *et al.* 2013; Soni *et al.* 2014). This study aims to investigate the therapeutic benefits of miR-132 overexpression on beta-cell function *in vivo*. In order to overexpress miR-132 specifically in beta-cells, we created an adeno-associated virus (AAV) vector containing miR-132 under control of the insulin promoter. AAV gene transfer has previously shown to efficiently and stably transduce beta-cells *in vivo* without impacting beta-cell function (Wang *et al.* 2006; Montane *et al.* 2011). Subsequently, the impact of miR-132 overexpression on beta-cell function was studied in mice under normal and insulin resistant conditions.

4. Material and Methods

Generation of viral vectors The dsAAV8-RIP-GFP vector was constructed on the backbone of the dsAAV8 plasmid (Nathwani *et al.* 2006). The original LP1 promotor and FIX coding sequences were replaced by the rat insulin promotor (RIP) (Addgene Plasmid 15029) and enhanced green fluorescent protein (eGFP), by amplification using primers in table 1 and using *MscI-BstXI* and *EcoRI-HindIII* restriction sites. The gene encoding miR-132 was amplified from chromosomal DNA from C57Bl/6J mice, using primers listed in table 1 and cloned between the RIP and eGFP in dsAAV8-RIP-GFP, using the restriction enzymes *BspEI* and *SbfI* (New England Biolabs, Ipswich, MA, USA).

AAV creation dsAAV viral particles were generated by triple transfection of human embryonic kidney 293 cells using the 25-kDa linear polyethylenimine (Polysciences Inc., Eppelheim, Germany) transfection method (Reed *et al.* 2006). AAV viral particles were purified by iodixanol gradient centrifugation as previously described (Zolotukhin *et al.* 1999). Viral titers were determined using quantitative PCR (qPCR) with primers specific for RIP and eGFP.

Mice and in vivo virus injection Male C57Bl/6J wildtype, ob/ob and ob/+ mice (9-10 weeks) were obtained from the Harlan Laboratories. C57Bl/6J mice were injected with AAV at the age of 12 weeks. Intraductal injection was performed as described (Jimenez *et al.* 2011) with minor adjustments. Mice were anaesthetized with isoflurane. The duodenum was isolated with the common bile duct attached. A microclamp was placed on the bile duct caudal to the liver. Using a 27G needle, the duodenum was punctured after which the needle was inserted to advance retrograde through the sphincter of Oddi into the common bile duct. The needle was secured in place using a ligation and 100 µl PBS containing $1.4x10^{11}$ viral genome particles was injected into the duct over approximately 1 min. At 1 min post-injection the

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96 microclamps and needle were removed. The puncture in the duodenum was closed using
97 tissue glue. Mice received buprenorphine (0.05 mg/kg s.c.) directly and 8 hours after surgery.
98 Mice were allowed to recover for 2 weeks on chow diet (RMH-B, Hope Farms, Woerden, the
99 Netherlands), after which mice received chow or high fat diet (60% kcal% fat diet, #D12492,
100 Research Diets Inc., New Brunswick, USA) for 4 weeks. All experiments were performed
101 with the approval of the Ethical Committee for Animal Experiments of the University of
102 Groningen.

103 *Primary mouse islet isolation, cell culture and in vitro insulin secretion assay*. Islets were
104 isolated by collagenase digestion as previously described (Salvalaggio *et al.* 2002). Islets
105 were rinsed and handpicked in RPMI media containing 10% FBS after which islets were
106 frozen immediately for RNA isolation or cultured overnight. The following day, static insulin
107 secretion assay was performed on size matched islets as previously described (Brunham *et al.*
108 2007). Insulin was measured by ELISA (Mouse-Insulin Ultra Sensitive ELISA or the Rat
109 Insulin ELISA, Alpco, Salem, NH, USA). Rat insulin-producing INS-1E cells (provided by
110 Dr. P. Maechler, Centre Médical Universitaire, Geneva, Switzerland) were cultured as
111 previously described (Merglen *et al.* 2004). INS-1E cells of passage numbers 50-60 were used
112 in our experiments. For transfection, INS-1E cells were transfected using lipofectamine 2000
113 (Invitrogen, Carlsbad, CA, USA) with miRNA mimics (Ambion, Life Technologies, Eugene,
114 OR, USA). At 2 days after transfection, glucose stimulated insulin secretion was measured.
115 To determine whether hyperglycemia would impact miR-132 expression levels, primary
116 mouse islets were cultured for 24 hours in RPMI containing 2 mM, 8 mM or 16 mM glucose
117 with 1% BSA with or without 0.4 mM palmitate (Sigma-Alrich, St. Louis, MO, USA).
118
119 *Glucose tolerance test and BrdU labeling* Oral glucose tolerance tests were performed on 8-
120 hour fasted mice administered with 2 g glucose per kg of body weight. Blood was taken by
121 the saphenous vain and blood glucose levels were measured using a glucometer and test strips
122 (Life Scan). For plasma insulin levels, blood was collected using EDTA-coated capillary

tubes and insulin levels were measured by ELISA (Mouse-Insulin Ultra Sensitive ELISA, Alpco, Salem, NH, USA). For beta-cell proliferation measurements, mice were daily injected i.p. with 1 mg/animal BrdU (Sigma-Aldrich) in PBS for 4 days before sacrifice.

Immunostaining and islet morphology analysis Formalin-fixed pancreatic tissues were embedded in paraffin using standard techniques. 4-µm sections were deparaffinized, rehydrated, and incubated with blocking solution. Sections were incubated overnight at 4° C with antibodies against insulin and glucagon (Dako, Glostrup, Denmark), GFP (Life Technologies, Eugene, OR, USA), and/or BrdU (Abcam, Cambridge, UK), followed by secondary antibodies conjugated to FITC or Cy3 (Life Technologies). DAPI-containing mounting media (Vector Laboratories, Burlingame, CA, USA) was added to coverslips. Proliferating beta cells were identified by co-staining for BrdU and insulin. All islets in 2 pancreatic sections of 200 µm apart were analyzed, resulting in the counting of at least 573 beta-cells/mouse with $n = 4$ mice per group. For beta cell area measurements, the percentage of insulin-positive area was determined using ImageScope (Aperio) from 5-6 evenly spaced sections per pancreas.

Measurement of miRNA and mRNA expression Total RNA from isolated islets was isolated using the mirVana kit (Life Technologies) according to instructions. RNA quality and concentration was measured using the Bio-rad Experion Bioanalyzer and miRNA microarrays were run with miRNA microarrays (MirBase release 17.0) of Agilent (Santa Clara, CA, USA). Array images were analyzed using Agilent feature extraction software (10.7.3.1) and GeneSpring GX (Agilent). After quantile normalization, statistical significance was tested with an unpaired t test followed by Benjamini-Hochberg multiple testing correction [false discovery rate: 0.01 and a fold change of at least 2]. cDNA for miRNA expression measurements was synthesized using Taqman miRNA reverse transcription kit (Life Technologies). For mature miRNA transcript expression, we used Taqman miRNA Assays

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(Life Technologies). cDNA for mRNA expression measurement was synthesized using Superscript II (Life Technologies). SYBR Green PCR Master Mix (Life Technologies) was used for RT-PCR in an ABI Prism 7700 Sequence Detection System. Expression values were normalized to GAPDH for mRNA and small nucleolar (sno) RNA202 for miRNA qRT-PCR.

Protein analysis Isolated islets were lysed in M-PER lysis buffer (Thermo Fisher Scientific, USA). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using TBST with 2% milk and 0.5% BSA for 1hr at room temperature followed by incubation with the primary antibodies in TBST with 0.5% BSA for 2 hours. Primary antibodies used were anti-retinoblastoma (Santa Cruz Biotechnology), anti-Cact (Novus Biologicals), anti-actin (Sigma Aldrich) and anti-Gapdh (Milipore). After probing with the primary antibodies, the membrane was incubated with HRP-conjugated secondary antibodies (rabbit-anti-mouse HRP conjugated, or goat-anti-rabbit HRP conjugated, Dako, Denmark). Chemilunimescence was determined using SuperSignal West Dura Extended Duration Substrate buffer (Thermo Fisher Scientific) and Chemidoc (Biorad, USA).

Statistical Analysis Graphpad Prism 6.0 was used for statistical analysis. Data are presented as Tukey's Box-and-Whiskers plot using median and 25^{th} and 75^{th} percentile intervals (P_{25}-P_{75}). Differences between groups were calculated by Mann-Whitney test with a P value of 0.05 considered significant. A two-way ANOVA, followed by Bonferroni posthoc tests, was used to evaluate the glucose tolerance tests.

5. Results

Leptin deficiency resulted in profound differences in miRNA profiles of ob/ob islets, with 36 miRNAs increased and 36 miRNAs decreased by >2 fold compared to control islets (n=6, FDR<1%). MiR-132 was one of the most induced miRNAs in ob/ob islets with a fold change of 6.2 (Fig 1A). Using RT-PCR, we showed increased expression of miR-132 in islets of ob/ob mice, high fat fed mice and 14-month aged mice (Fig 1B). As all the diabetic/insulin resistant models tested had elevated fasting blood glucose levels, we determined whether hyperglycemia itself regulated expression of miR-132. Indeed, miR-132 expression levels were upregulated by culturing primary mouse islets in 16 mM glucose for 24 hours. This increase was augmented by the addition of 2 mM palmitate (Fig 1C). In agreement with previous data (Nesca *et al.* 2013; Soni *et al.* 2014), overexpression of miR-132 in INS1E cells resulted in increased glucose-stimulated insulin secretion (Fig 1D). Together these results confirm previous data showing a role for miR-132 in glucose-regulated insulin secretion in beta cells *in vitro*.

Increased miR-132 expression result in improved insulin secretion during high fat feeding

To determine whether increased expression of miR-132 would increase insulin secretion *in vivo*, we created double stranded adeno-associated virus serotype 8 (dsAAV8) vectors containing mouse miR-132 and GFP cDNA or GFP cDNA driven by the rat insulin promoter (AAV8-RIP-mir132 and AAV8-RIP-GFP). Injection of the constructs in the pancreatic duct resulted in GFP expression specifically in β-cells in the islet (Fig 2A). Based on the GFP, the proportion of islets with GFP positive cells was relatively high; 77±15 % for control and 88±5% for AAV-RIP-miR132 treated mice. Increased expression of miR-132 was detected in isolated islets of AAV-RIP-miR132 injected animals, confirming miR-132 overexpression in β-cells *in vivo* (Fig 2B). Histological examination of the pancreata of the mice showed no evidence of pancreatitis or fibrosis. Islets of both groups displayed normal morphology with

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the β-cells in the core and the α-cells at the rim of the islet (Fig 2E).

Therapeutic overexpression of miRNAs could potentially modify the processing of other cellular miRNAs transcripts due to the same processing pathways (Grimm *et al.* 2006). Therefore, we tested the expression of miR-375, a microRNA highly expressed in beta cells and involved in regulation of insulin secretion (Poy *et al.* 2004) and miR-184, a modulator of compensatory beta cell expansion during insulin resistance (Tattikota *et al.* 2014). To exclude a possible negative feedback loop due to miR-132 overexpression on the miR-132/miR-212 cluster, miR-212 levels were determined in the islets of control and miR-132 treated mice. Overexpression of miR-132 did not impact expression of miR-212, miR-375 or miR-184 (Fig 2C), indicating that overexpression of miR-132 did not interfere with the processing of other microRNAs.

After initial weight loss in the first week after the injection, all mice gained weight 1 month after the injection (Fig 3A) after which glucose homeostasis was analyzed. Surprisingly, overexpression of miR-132 did not impact fasted glucose or insulin plasma levels. In addition, glucose tolerance testing showed no difference in glucose control between the animals (Fig 3B,C). To increase the demand on beta cells, mice were put on a high fat diet for 4 weeks. In the control AAV-RIP-GFP treated mice, high fat diet increased fasted glucose levels and impaired glucose tolerance (Fig 3D). Overexpression of miR-132 specifically in beta cells, however, resulted in improved glucose tolerance compared to control mice. This was due to increased glucose-stimulated insulin secretion as measured during the glucose tolerance test (Fig 3E) and *ex vivo* in isolated islets (Fig 3F). Insulin content of isolated islets was similar in control and AAV-RIP-miR132 treated mice (0.37±0.04 μg/islet in control islets *vs*. 0.33±0.04 μg/islet in miR-132 islets; n=6). In addition, gene expressions of genes related to insulin secretion or beta-cell function, such as *insulin*, *glucose transporter 2* (*Glut2*), *prohormone convertase 2* (*Psck2)*, *MAF bZIP transcription factor A* (*Mafa)* or *pancreatic and duodenal homeobox 1* (*Pdx1)* were similar between groups (Fig 3G). Gene expression and protein levels of the miR-132 target carnitine acyl-carnitine translocase (Cact) (Soni *et al.* 2014) was

decreased in islets of AAV-RIP-miR132 injected animals (Fig 3H).

Increased beta cell proliferation in mice overexpressing mir-132

Overexpression of miR-132 in dispersed rat islet cells has been shown to increase beta-cell proliferation *in vitro* (Nesca *et al.* 2013). In order to determine whether overexpression of miR-132 *in vivo* also lead to increased beta cell proliferation, beta cell proliferation was studied in the AAV-RIP-miR132 and control mice after 2 weeks of high fat diet feeding using BrdU incorporation. Overexpression of miR-132 lead to 2.4-fold increase in $BrdU^+$ beta cell (Fig 4A,B). In agreement with this, we found increased expression levels of the proliferation marker *Ki67* in isolated islets from AAV-RIP-miR132 mice fed a high fat diet for 4 weeks (Fig 4C). Although, gene expression levels of the previously identified miR-132 target *retinoblastoma protein (Rb)* (Park *et al.* 2011) were similar in both groups (1.0±0.5 relative expression in control islets *vs*. 1.2±0.3 relative expression in miR-132 islets; p=0.48), protein analysis revealed decreased Rb protein levels in islets of miR-132 treated mice (Fig 4 D). To determine whether the increased proliferation resulted in increased beta cell mass, beta cell area was analysed. However, the beta cell area did not significantly differ between the groups (0.84±0.29% beta cell area in control islets *vs*. 1.18±0.38 % beta cell area in miR-132 islets; n=4; p=0.2).

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6. Discussion

Our data show that AAV8-mediated gene transfer of miR-132 to beta cells improves beta cell function in mice in response to a high fat diet. We found significant increased glucose-stimulated insulin secretion and enhanced beta cell proliferation in mice treated with the dsAAV8-RIP-miR132 construct. These data indicate that miR-132 is a potential target to improve beta cell dysfunction during obesity.

Although, overexpression of miR-132 improves insulin secretion *in vitro*, mice glucose homeostasis remained unaltered in chow-fed mice overexpressing miR-132 indicating that the risk on hypoglycaemia is low. Mice treated with dsAAV-RIP-miR132, however, did show improved insulin secretion during high fat diet feeding. Recent findings identified Cact as miR-132 target in beta cells (Soni *et al.* 2014). Cact is a transporter involved in transporting long-chain acyl-carnitines into the mitochondria for β-oxidation (Wang *et al.* 2011). Downregulation of Cact results in an accumulation of fatty acyl-carnitines, which enhances insulin secretion. Addition of long-chain acyl-carnitines to beta cells stimulated insulin secretion; an effect that was enhanced by Cact downregulation (Soni *et al.* 2014). This could explain our finding that only during increased fatty acid influx, as achieved by the high fat diet, insulin secretion was increased in the miR-132 overexpressing beta cells.

In this study we chose to apply beta cell specific gene therapy to evade possible side effects of miR-132 overexpression in non-beta cells. MiR-132 has previously found to be involved in facilitating pathological angiogenesis in tumors (Anand *et al.* 2010) and is over-expressed in pancreatic adenocarcinomas (Park *et al.* 2011). In the pancreatic cancer cell line PAN-1, overexpression of miR-132 leads to decreased expression of the tumor suppressor Rb, leading to increased proliferation (Park *et al.* 2011). No difference in expression of *Rb* mRNA in islets of dsAAV8-RIP-miR132 treated mice was found, which could be due to the relative high gene expression of *Rb* in alpha cells compared to beta cells (Kutlu *et al.* 2009). Protein levels of Rb, however, were decreased in islets overexpressing MiR-132. Interestingly, exendin-4, a GLP-1 agonist known to induce beta cell proliferation in mice, has been shown

to decrease Rb expression. Further study revealed that this decreased Rb expression is necessary for the beta cell proliferation stimulating effect of excendin-4 (Cai *et al.* 2014). GLP-1 agonists increase miR-132 expression in beta cells (Shang *et al.* 2015), suggesting that miR-132 plays an central role in the adaptive beta cell response to obesity and GLP-1. Although we found increased BrdU incorporation and increased Ki67 expression in islets of dsAAV8-RIP-miR132 treated mice after high fat feeding indicating increased beta cell proliferation, beta cell area was not significantly different between the groups. The high variation in beta-cell area within the groups, the small group size and the relatively short period of high fat diet could potentially explain this discrepancy.

Unfortunately, it was not possible to identify miR-132 targets in our setting due to the difficulty to isolate pure beta cells together with the fact that miRNAs often induce only small changes in the expression of single direct targets (Guo *et al.* 2010). However, our study does show that the physiological impact of miRNAs in beta cells can be successfully studied *in vivo* using the AAV8-mediated gene transfer system. This system could potentially help to identify the physiological roles of the over 800 miRNAs which recent ultra-high-throughput sequencing have revealed to be expressed in the endocrine pancreas (Kameswaran *et al.* 2014).

During the last years, the importance of microRNAs in the control of beta cell function, proliferation and identity has become clear. Several microRNAs, such as, miR-375 and miR-184 have been identified as crucial regulators of adaptive beta cell expansion, whereas miR-7a regulates insulin secretion. Our study shows the beneficial effects of miR-132 overexpression in the setting of obesity and identifies miR-132 or its downstream targets as therapeutic targets to improve beta cell function.

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298 **7. Declaration of interests:** The authors have no conflicts of interest to declare.

299

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303

304 **9. Author contributions:** NLM, JK and JKK designed the experiments. NLM, RH and
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306 authors revised the article and approved the final version.

307

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Figure legends

Figure 1. **Hyperglycemia and obesity induced miR-132 expression resulting in increased insulin secretion.** A. Several miRNAs were differently expressed in pancreatic islets of 12 week old ob/ob mice (n=6). B. Increased miR-132 expression in islets isolated from ob/ob (n= 4), 10 week high diet-fed C57Bl6 mice (n=4) or 14 month old C57Bl6 mice (n=7-8). C. Culture of primary islet for 24 hours in high glucose media or media containing 2 mM palmitate induced miR-132 expression (n=4). D. Overexpression of miR-132 in INS1E cells resulted in increased glucose-stimulated insulin secretion (n=4).

Figure 2. **AAV8 mediated gene transfer resulted in miR-132 overexpression in beta cells.** A. Representative image of immunofluorescent staining against GFP (green) and insulin (red) in control AAV-RIP-GFP or AAV8-RIP-miR132 treated mice. B. Increased expression of miR-132 in islets isolated from AAV8-RIP-miR132 treated mice compared to islets isolated from control mice (n=4-6). C. Expression of miR-375, miR-184 and miR-212 in islets was comparable between AAV8-RIP-miR132 treated and control mice (n=4-6). D. Representative image of islet morphology based immunofluorescent staining against insulin (green) and glucagon (red).

Figure 3. **Impact of miR-132 overexpression in beta cells on glucose homeostasis.** A. Body weights of AAV8-RIP-GFP control and AAV8-RIP-miR132 treated mice (n=6) during chow and high fat diet (HFD) feeding. B. AAV8-RIP-miR132 treated mice showed similar glucose levels during fasting or after oral glucose bolus as control mice fed a chow diet (n=6). C. Insulin levels at 0 and 15 minutes after oral glucose bolus were similar between the 2 groups (n=6). D. Oral glucose tolerance testing showed improved glucose tolerance in AAV8-RIP-miR-132 treated mice after 4 weeks of high fat diet (n=6). E. Analysis of insulin levels at 0 and 15 minutes after oral glucose bolus showed increased insulin secretion in mice overexpressing miR-132 after the glucose bolus (n=6). F. Ex vivo analysis of glucose-

stimulated insulin secretion showed increased insulin secretion at 16.7 mM glucose in miR-132 overexpressing islets (n=6). G. Isolated islets of AAV8-RIP-miR132 treated mice showed normal gene expression of beta-cell related genes (n=4-6). H. Increased expression of miR-132 coincided with reduced expression of CACT mRNA and protein levels (n=4-6).

Figure 4. **Signs of increased proliferation in beta cells of AAV8-RIP-miR132 treated mice.** A. Pancreatic sections of control or AAV8-RIP-miR-132 treated mice stained using immunofluorescence for insulin (green) and BrdU (red). B. Percentage of BrdU positive beta cells in pancreata of control and AAV8-RIP-miR-132 mice (n=4). C. Isolated islets of AAV8-RIP-miR132 treated mice showed increased Ki67 gene expression (n=4-6). D. Decreased Rb protein levels in isolated islets of AAV8-RIP-miR132 treated mice, of which the quantification is shown in E (n=6).

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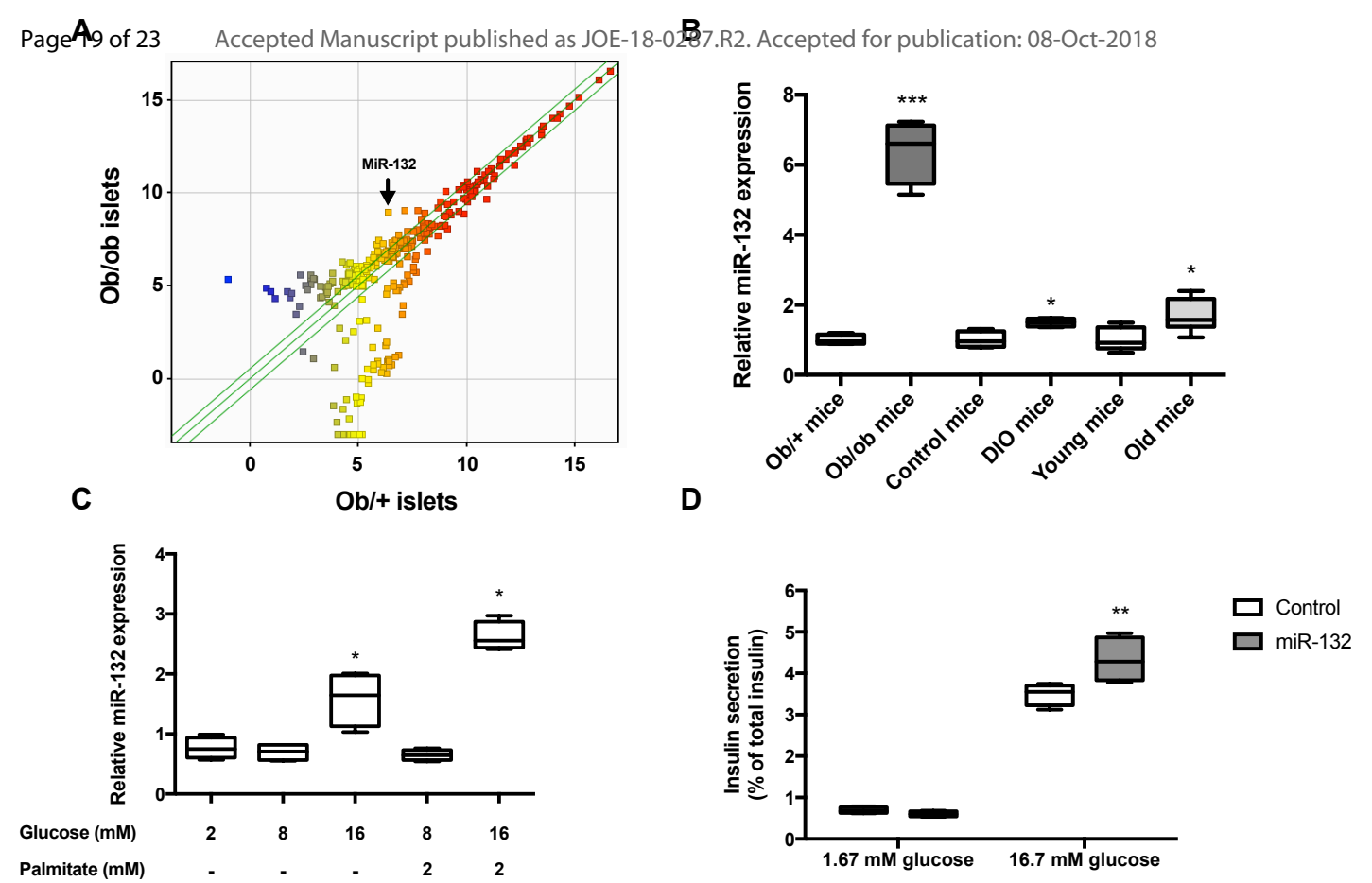

Figure 1

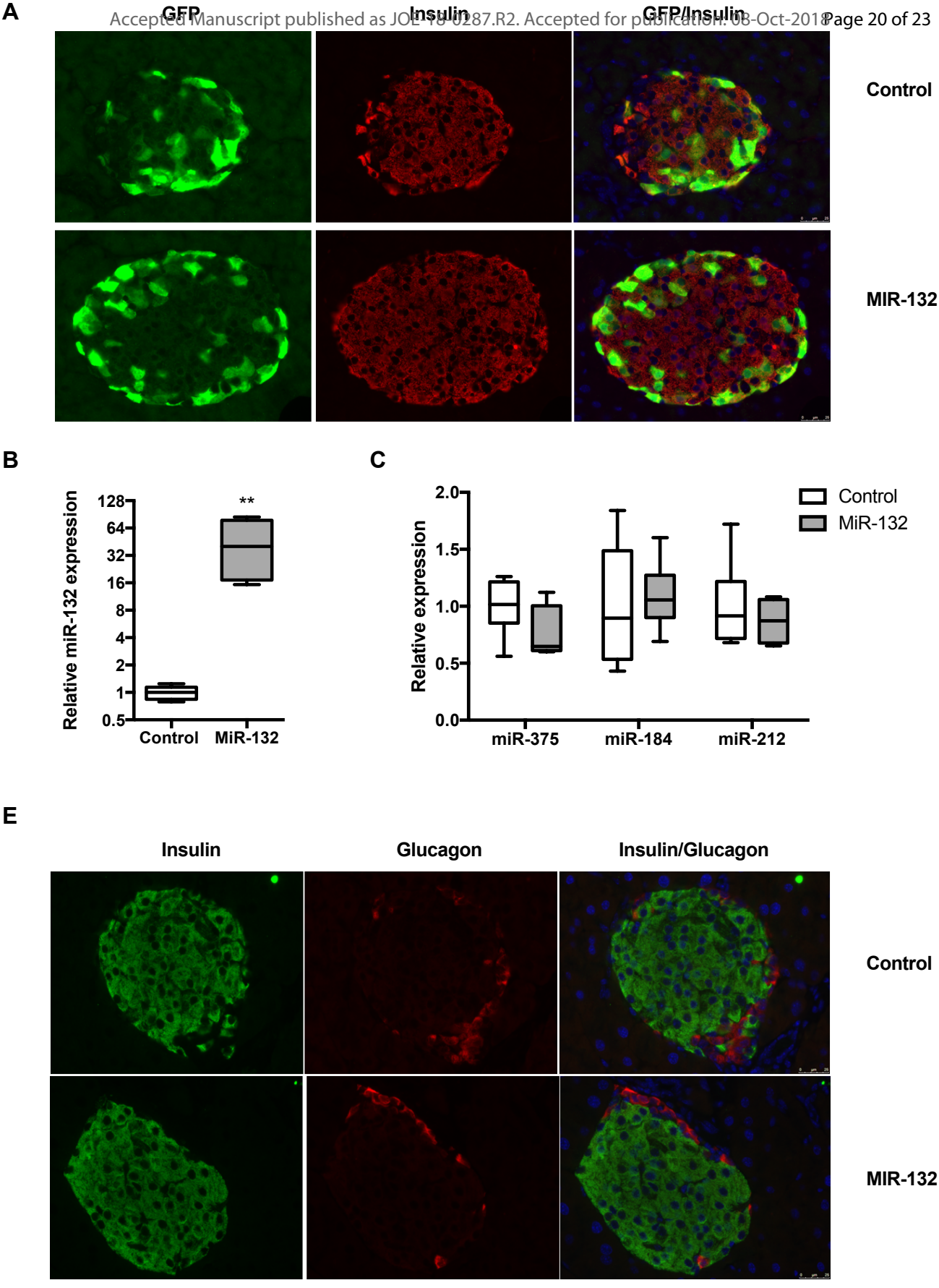

Figure 2

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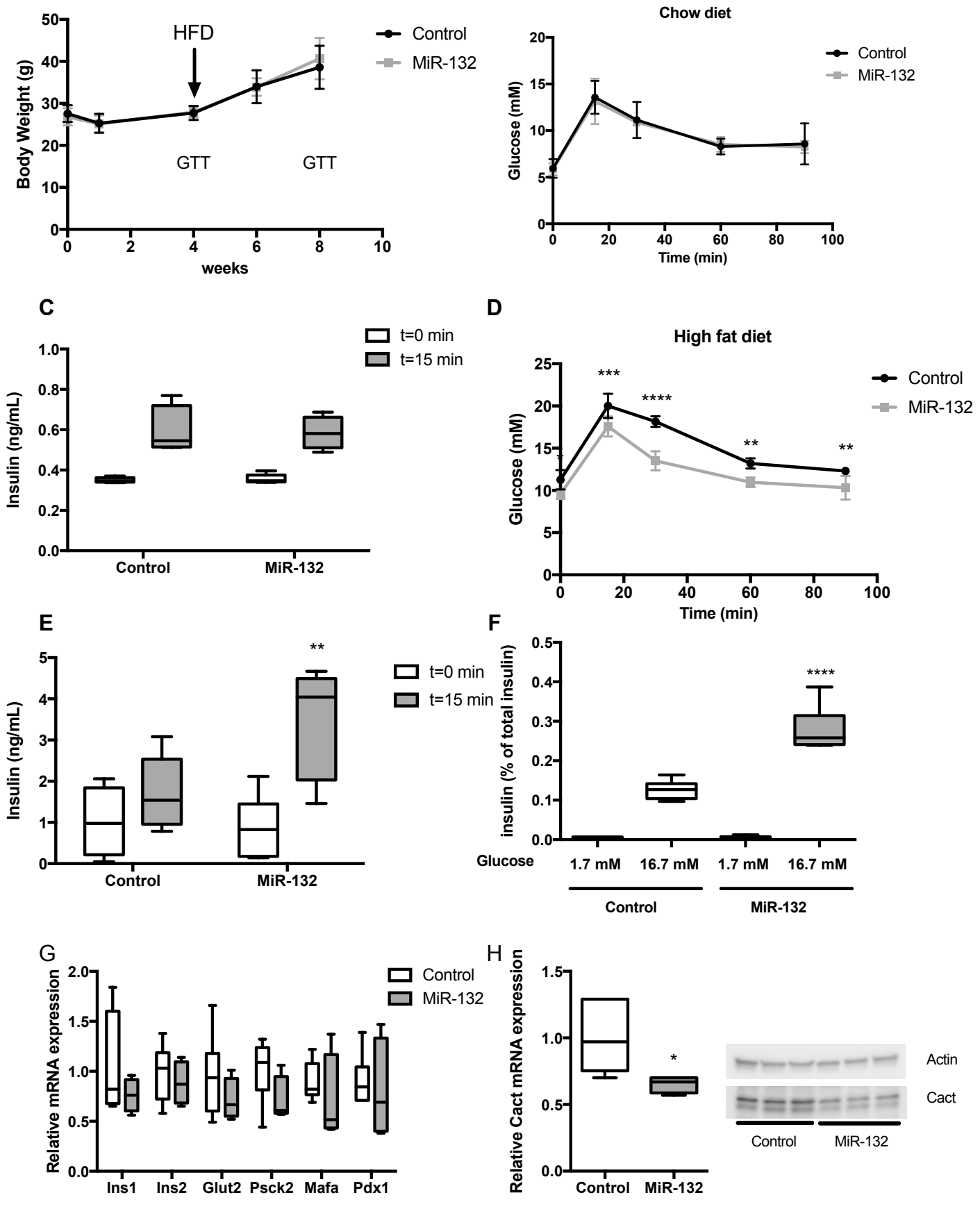

Figure 3

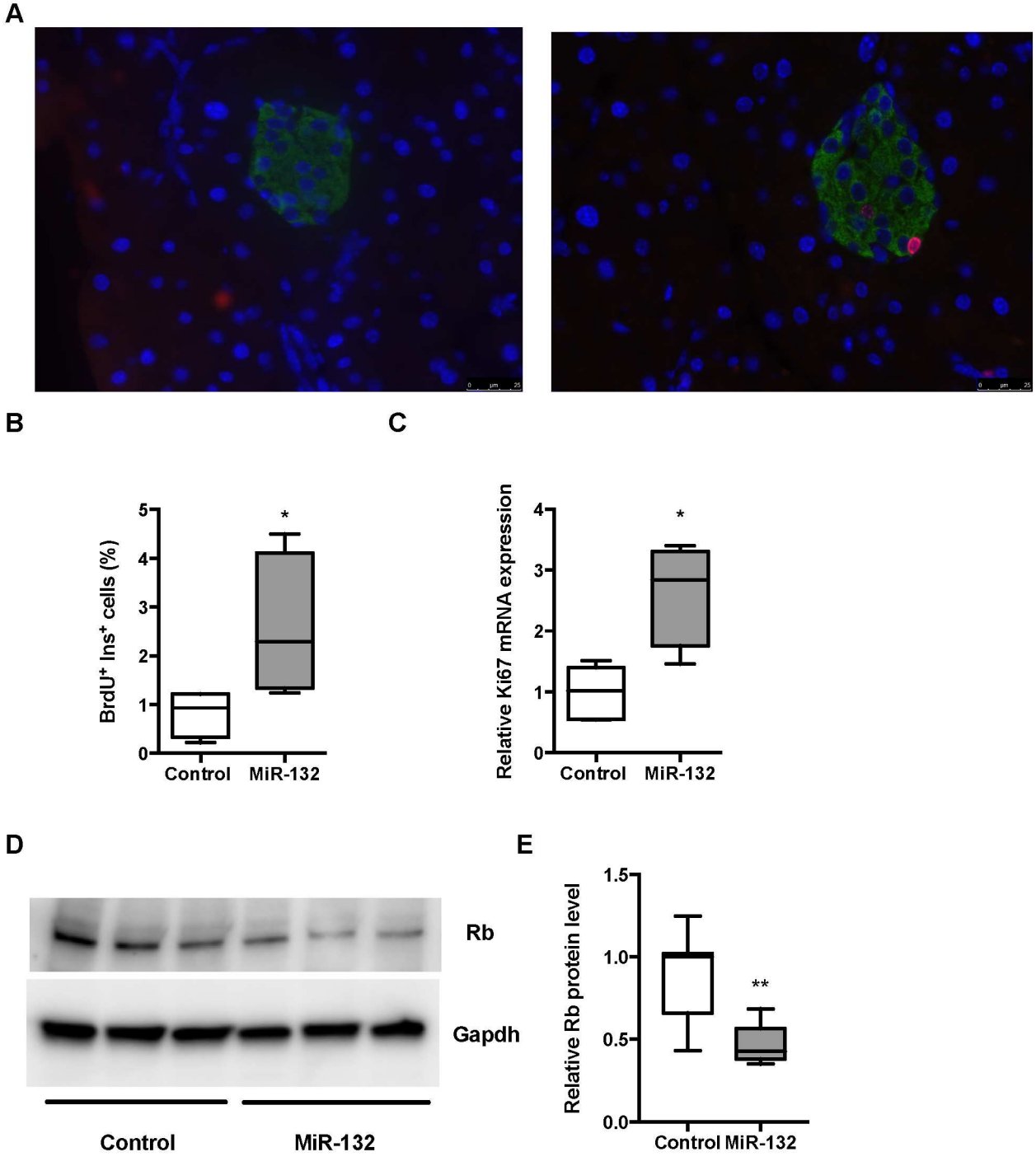

Figure 4

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13. Tables

Table 1: Primer sequences

Primer Name	Sequence	Gene
scAAV-GFP-fw	tactacgaattcaccatggtgagcaagggcgag	GFP
scAAV-GFP-rv	tactacaagctttcacttgtacagctcgtcca	GFP
scAAV-RIP-fw	gtggagtcgtcgtaccgggccc	RIP
scAAV-RIP-rv	gttgccaggtcagtgggcatgcctgc	RIP
Mmu-mir132-fw	gcgaaacctgcaggtccctgcgccgctgtccgcg	Mmu-mir132
Mmu-mir132-rv	gcgaaatccggatgccacctccgcagacacat	Mmu-mir132